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The effects of Flouride Ion on some Blood Constituents of Rainbow Trout, Salmo gairdneri, Linnaeus

Richard H. Alger

THE EFFECTS OF FLUORIDE ION ON SOME BLOOD CONSTITUENTS OF RAINBOW TROUT, *Salmo gairdneri* Linnaeus

by

Richard H. Alger

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To

E. M. R. L.

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INTRODUCTION

During the past quarter of a century there has been considerable investigation into the effects of fluorides on living organisms. It has been well established, as a result of these studies, that both small and elevated amounts of fluorides present in the environment may have a marked toxic effect upon gaining entry into the organism. Much of the research involving fluorides and the living organism has been confined to experimentation with animals, although considerable evidence indicates that plants are also subject to injury if fluorides are present in the atmosphere or the soil.

The great majority of the research dealing with effects of fluorides on animals has been confined to higher vertebrates, principally domestic farm species. The concentration of fluoride research in this area has been prompted by economic pressures arising from natural, industrial, or accidental fluoridation of livestock. For the most part, these investigations have been confined to determination of toxicity levels to various domestic species, while the mode of fluoride action upon living organisms has often been neglected. Some investigators, however, have postulated "defense mechanisms" within the organism whereby active fluoride ion is removed from systemic circulation and is either deposited in less active tissues of the body, or, is excreted in some manner. Such mechanisms would offer a degree

of temporary protection of the organism from harmful effects of the fluoride ion.

Within recent years, with the immediate economic problems of fluoridation having been allayed to a considerable degree, there has been a tendency toward basic research in fluoride investigations. This tendency has been manifested in the design of experiments to seek out the nature and mechanism of fluoride action in the organism. Although earlier workers had suggested the possibility of enzyme inactivation, results of recent research indicate that the primary point of attack of fluorides is on enzyme systems of the organism. It may be that there is a gradual, cumulative destruction of enzymes whereby the enzyme-protein complex combines with fluoride ion and, as a result, is altered in some manner. It has been shown that fluoride ion forms stable complexes with certain divalent cations which are required to activate some enzymes. These processes may bring about a gradual cessation of metabolic reactions upon which life depends.

Within very recent years, research has been directed toward assessing the effects of fluorides on some wildlife species. Knowledge that naturally or industrially fluoridated waters may have deleterious effects on aquatic biota has stimulated research in this area. Thus far, research in this realm has dealt primarily with the effects of fluorides on several species of freshwater fishes. The ultimate concern of these projects is the accumulation of sufficient knowledge to assess accurately the effects of fluorides in aquatic environments on fish populations.

In my study, the effects of an artificially fluoridated

medium on serum protein levels, serum protein fraction concentrations, alkaline phosphatase activity of the serum, and serum concentrations of calcium and magnesium ions were investigated in the attempt to uncover some of the effects that fluoride ion has on the rainbow trout, Salmo gairdneri Linnaeus.

REVIEW OF LITERATURE

The literature elucidating the modes of fluoride action on the physiology of the fish is notable for its relative scarcity as compared to the voluminous material dealing with the effects of fluorides on higher vertebrates.

Neuhold (1959) found that there were significant changes in the electrophoretic mobilities of three protein fractions in the serum of carp exhibiting symptoms of acute fluoride intoxication. Ten protein fractions were found in carp serum. Two fractions showed increased levels in the fluoridated carp, while one fraction exhibited a decline in percent composition of total serum protein. Tetanic seizures, an increase in the density of mucous cells on the head region and on the epithelium of the gill lamellae, and hypertrophy of the ultimobranchial gland possibly associated with decreased blood calcium levels were reported in fluoridated rainbow trout. Uptake of fluoride by cancellous and skeletal bone was proportional to the concentration of fluoride ion in the medium, and it was suggested by Neuhold (op. cit.) that uptake of systemic fluoride by osseous tissue marks a defense mechanism whereby active fluoride ion is removed from circulation in the fish and is deposited in the form of a stable mineral complex.

De Roos (1958) reports an increase in the number of mucous cells on the gill lamellae of goldfish held in fluoride ion concentrations of 65, 80, and 114 ppm. This is accompanied by

edema of lamellar epithelial cells, with a possible hypertrophy of certain inter-lamellar cells which are associated with chloride excretion.

That such "chloride cells" may have an excretory function is suggested by alkaline phosphatase activity around these cells in the gill lamellae of Fundulus; it is suggested by Pettengill and Copeland (1948) that these chloride cells have an excretory function in salt and brackish waters, but serve to absorb ions in fresh water, thus aiding in the maintenance of a systemic osmotic balance.

Neuhold (1959) postulates that development of mucous cells on the gill lamellae and on the head region in response to elevated levels of fluoride ion in the medium marks a defense mechanism whereby active fluoride is excreted from the system of the fish.

Phillips (1932) found that plasma phosphatase activity in fluoridated cattle rises in proportion to the level of fluoride ingested. Phillips (1934) appears to be the first investigator to suggest that increased phosphatase activity in the fluoridated animal may serve as a mechanism whereby fluoride ion is removed from systemic circulation and deposited in osseous tissue. It is suggested (Phillips, op. cit.) that "... fluorine toxicosis produces its systemic reaction through interferences with cellular respiration ...the primary point of attack is the enzyme system of the body..."

Reviews of the literature pertaining to effects of fluorides on animals have been presented by McClure (1933), DeEds (1933), Roholm (1937), Pierce (1939), Greenwood (1940), Mitchell and Edmans (1945), and Greenwood (1956). A bibliography of fluoride literature has been prepared by Campbell, Christian, and Widner (1950, 1953).

The alkaline phosphatases are a group of enzymes having wide distribution in both plant and animal tissues. Discussions of the various functions of this group are found in Hawk, Oser, and Summerson (1951), Fruton and Simmonds (1953), Best and Taylor (1955), Turner (1955), Brown (1957), and Harrow and Mazur (1958).

The alkaline phosphatases are hydrolytic phosphomonoesterases which catalyze the hydrolysis of monoesters of phosphoric acid (e.g., alpha-glycerophosphate, p-nitrophenylphosphate, glucose-6-phosphate, etc.). The pH for optimum alkaline phosphatase activity is around 9.0. It is generally reported that Mg^{++} and Ca^{++} are necessary for the activation of alkaline phosphatase. Best and Taylor (1955) report that Ca^{++} has been found to be mildly inhibitory to alkaline phosphatase activities.

Alkaline phosphatase is present in greatest amounts in ossifying cartilage and is believed to be a product of the osteoclasts, proliferating cartilage cells, and the cells of the inner layer of the periosteum. It is postulated that these cells release alkaline phosphatase which, in the calcification process, liberates inorganic phosphates from phosphoric esters, creating a surplus of PO_4^{---} in the presence of Ca^{++} wherein the solubility product of $Ca_3(PO_4)_2$ is exceeded and the precipitation of calcium phosphate in osseous tissue occurs (Best and Taylor, 1955). Alkaline phosphatase appears to function in nutrient transfer across the intestinal mucosa, in glycolysis, and in excretory cells of the gills of certain fishes (Brown, 1957).

A marked rise in alkaline phosphatase activity is associated with certain diseases of the bone, hyperparathyroidism (Best and

Taylor, 1955), and is reported to rise in fluoridated animals in some cases. Alkaline phosphatase activity in the serum of fluoridated animals has been reported to rise (Phillips, 1932, 1934) in bovines, to be variable in bovines and rats (Du Toit et al., 1937; Majumdar, 1946), and to remain unchanged in chickens (Hauck et al., 1933), rats (Smith et al., 1935), and bovines (Olson et al., 1958).

Soulariac (1949) reports that NaF inhibits ATP activity in striated rat muscle. Danowski (1949) found that the rate of glucose disappearance in incubated blood (in vitro) decreases with increased fluoride ion concentration; the effects are partially or completely cancelled by addition of calcium or magnesium salts. Eichler (1949) reported 4 organic fluorides inhibit alkaline bone phosphatase activity. Kind et al. (1952) report that fluoride ion concentrations of 10^{-7}M. through 10^{-3}M. do not inhibit alkaline phosphatase activity in frog liver. Fluoride ion is reported to form stable complexes with Ca^{++} and Mg^{++} thus inhibiting those enzymes which require these cations as activators (Whittaker, 1954).

Various workers differ markedly on their findings as to the levels of Ca^{++} in the serum of fluoridated animals. Serum calcium is reported to rise in the rabbit (Bogdanovic, 1935), to fall in the chicken (Hauck et al., 1933), the rat (Irving, 1943, 1944, 1946), and in cattle (Majumdar, 1946; Leone et al., 1956). No changes in serum Ca^{++} levels were reported in fluoridated dogs (Greenwood et al., 1935) and cattle (Olson et al., 1958).

L. E. Olson[1] indicates that serum Mg^{++} values in fluoridated

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[1]Personal communication.

cattle range from 0.6 - 2.72 mg./100 ml. and that serum Mg^{++} rises proportionally with the level of fluoride ingested by the animal.

Field et al. (1943) report the mean value for serum Ca^{++} in normal carp is 11.50 mg./100 ml., with a range of 9.45 - 14.77 mg./100 ml. Serum Mg^{++} levels for normal carp are reported; the mean value is 3.32 mg./100 ml., with a range of 2.52 - 3.88 mg./100 ml.

The function of the parathyroid glands of higher vertebrates in calcium deficiency has been established (Turner, 1955). Recently, the ultimobranchial glands of certain fishes have been assigned a parathyroid-like function. Stress was induced in the fish, Astyanax, and distortion of the vertebral column caused by calcium loss and subsequent fibrosis of bone was observed. Degenerating tubules, calcium deposits, granular deposits, and fibrosis were found in the kidney (Brown, 1957). Neuhold (1959) found hypertrophy of the ultimobranchial glands in rainbow trout exhibiting symptoms of acute fluoride intoxication.

Serum protein values were determined for brook and brown trout by Phillips (1958). Total serum protein concentration for the brook trout has a reported mean value of 2.01 gm./100 ml., and for the brown trout is 2.04 gm./100 ml.

The concentrations of serum protein fractions of higher vertebrates reportedly may fluctuate in both normal and pathological conditions. The levels of the globulins and albumin may vary independently of each other or concordantly, with or without alterations in levels of total serum protein. Hemoconcentration results in abnormally high concentrations of total serum protein, particularly if there is no leakage of the smaller plasma protein molecules into

intercellular spaces (Best and Taylor, 1955).

The production of ACTH in stress reactions facilitates glyconeogenesis through the stimulation of the adrenal cortex and subsequent release of gluco-corticoid hormones (Turner, 1955). The presence of ACTH and 11-oxy-corticosteroids has been demonstrated in stressed fish (Astyanax) (Brown, 1957).

Scassellati-Sforzolini (1954) reports that all proteins studied, with the exception of lactalbumin, effectively fix fluoride ion from NaF. Protein molecules bear characteristic sites which may be either positively or negatively charged (Giese, 1958; Harrow and Mazur, 1958). That attachment of F^- to positively charged sites on serum protein molecules would alter electrophoretic migration patterns of the various fractions would appear possible.

MATERIALS AND METHODS

All experimental phases of this study were conducted at the Fluorine Laboratory of the Department of Wildlife Management, Utah State University, Logan, Utah.

The physical plant

All experimentation was conducted within 2 walk-in type, refrigerated cooling units built into the laboratory. The temperature within the cooling units could be thermostatically controlled and any desired temperature between 40° F and 60° F could be maintained. All experiments of this study were conducted at 55° F, maximum temperature variation being ± 1° F.

Twelve 20-gallon aquaria were placed on steel racks inside each cooling unit. Each aquarium was constantly illuminated throughout all experimentation by one 14-inch fluorescent tube and one 7 1/2-watt incandescent bulb. Each aquarium was provided with a glass cover to minimize the probability of fish leaping from the aquaria while in tetanus.

Aeration of the water was accomplished with electric air pumps, 2 pumps in each cooling unit. Poly-ethylene air lines were attached to the main air line by a series of regulating valves. Each aquarium received one air line from the main line. An air stone was attached to the immersed end of each air line in the aquaria. The pumps provided in excess of 30 cubic inches of air per minute to each aquarium. In event of an electrical power failure, an auxiliary

oxygen supplying unit would automatically be thrown into operation.

Water used in all experiments was forced through an ion exchange resin which served to remove calcium and magnesium ions, thus preventing reduction of calculated concentrations of fluoride ion through precipitation as CaF_2 or MgF_2. Two 300-gallon tanks were employed as holding and conditioning tanks for the fish prior to each experiment. It was found necessary to allow water to stand in the holding tanks for at least 24 hours prior to the introduction of trout. This period was sufficient to remove chlorine. The same procedure was employed with water placed in the 20-gallon aquaria.

Experimental animals

The rainbow trout was selected as the experimental subject. It was found that this trout, in order to supply the minimum amount of serum necessary for the chemical analyses, must be approximately 10 inches long. Trout were secured from the Logan Fish Hatchery of the Utah State Department of Fish and Game several days prior to initiation of each experiment. The trout were held for at least 24 hours prior to the beginning of an experiment in two 300-gallon tanks. This time was deemed sufficient to effect adaptation of trout to the water. The trout were not fed after their transfer from the hatchery.

Experimental design

The design utilized in all experiments was a split block in time, with fluoride concentrations arranged at random within each block.

Experimental fluoride ion levels

Concentrations of fluoride ion added to the experimental medium were 2, 4, 7, 13, and 25 parts of fluoride per million parts of water. The control employed in all experiments was Logan City water. This water has a fluoride ion concentration of 0.2 ppm. For each experiment, the above concentrations were distributed randomly in each cooling unit. In each unit, or block, there were 12 aquaria. Two aquaria in each block held the same fluoride ion concentration in each experiment. Thus, there were 2 each of 0.2, 2, 4, 7, 13, and 25 ppm of fluoride ion in each block, making a total of 4 aquaria holding the same fluoride ion concentration in each experiment.

Experimental procedure

Following introduction of desired fluoride ion levels into the aquaria at least 24 hours prior to initiation of an experiment, 2 trout were added to each aquarium. Thus, 48 trout were utilized in each experiment, 8 in each fluoride ion concentration and 8 trout in 0.2 ppm of F^-, the control level. A total of 5 experiments was run. Each experiment was 12 hours longer than the preceeding experiment. The first run was terminated at 12 hours, the second at 24 hours, the third at 36 hours, the fourth at 48 hours, and the last run at 60 hours.

At the termination of each experiment the trout were collected immediately. An oblique excision of the tail, from midway between the dorsal fin down to within 1/2-inch of the caudal fin on the ventral surface was made on the living fish. Prior to decaudation, each fish was wrapped in a paper towel to reduce contamination of the blood sample by mucus. Following decaudation, the blood was

collected immediately into 15 ml. centrifuge tubes. Extreme care was taken to compress the trout as little as possible while the sample was being collected.

The blood samples were allowed to stand for 3 hours following collection. The samples were then centrifuged for 20 minutes at 2500 rpm. Following centrifugation, the serum was withdrawn from the centrifuge tubes with 3-inch, 18 gauge spinal injection needles attached to hypodermic syringes. The serum was transferred from the syringe to 78 mm. test tubes. The test tubes had been previously sterilized in an autoclave at 15 p.s.i. for 25 minutes. Following insertion of the serum into the test tubes, corks, previously sterilized by exposure to absolute alcohol vapors, were immediately inserted into the test tubes. The test tubes were then sealed with a coating of paraffin which was applied over the cork and the upper 1/3 of the tube.

After the samples were sealed in test tubes, those samples to be analyzed for total protein and those to be studied electrophoretically were immediately frozen. Samples utilized in electrophoretic analysis were frozen for approximately 5 weeks, while those samples to be examined for total protein content were frozen for 8 months. Leone (1949) reports that there is no change in the serological acuity of samples after 15 years of freezing. Those tubes containing serum samples to be analyzed for alkaline phosphatase activity were placed in a laboratory refrigerator and stored for no longer than 24 hours at 42° F.

In addition to trout sacrificed in perfection of experimental techniques and procedures, a total of 240 trout was utilized in

this study. A total of 48 trout was sacrificed in each experiment. Thus, there were 240 serum samples upon termination of all experimentation.

Chemical analyses

Determination of alkaline phosphatase activities was accomplished by utilization of the method of Bessey, Lowry, and Brock (1946). The authors suggest that the most accurate results are obtained if determinations are made within 3 days after the sample is secured. In all instances in my experiments, alkaline phosphatase determinations were made within 36 hours following collection of the serum samples. Standard curves were first prepared by utilizing procedures suggested in the technique. A Bausch and Lomb "Spectronic 20" colorimeter-spectrophotometer was employed in determination of serum alkaline phosphatase activities.

Determination of total serum protein followed the method of Gornall, Bardawill, and David (1949), a modified biuret technique. A Bausch and Lomb "Spectronic 20" was used. Standard curves were prepared utilizing Armour's standard albumin.

Separation of serum protein fractions was accomplished with a Spinco paper electrophoresis chamber. Following a 14-hour period in the electrophoresis chamber, the paper strips were processed and were then analyzed in a Spinco "Analytrol," which recorded the results on graph paper in the form of a series of peaks corresponding to the various serum protein fractions. The graphs were analyzed and the separate peaks were recorded as percent of total serum protein. The percentage composition of each peak, as determined by analysis of the graphs, was multiplied by the total serum protein value determined for

each fish. In this manner, values, expressed in grams of protein per 100 ml. of serum (gm./100 ml. or gm. %) were obtained for each serum protein fraction.

Concentrations of serum Ca^{++} and Mg^{++} were determined on the Beckman Model DU spectrophotometer with flame and photomultiplier attachments. The technique used in determination of calcium and magnesium was that of Teloh (1958). Standard curves were prepared with standard calcium and magnesium solutions alone, then again with standard solutions containing serum from control rainbow trout as an internal standard.

Prior to all chemical analyses, 2 serum samples per fluoride ion concentration in the blocks were selected at random from the total number of serum in each experiment. Thus, sera of 2 trout from each fluoride ion level were selected at random, making a total of 12 samples from each experiment, or, 60 serum samples from all experiments. Each serum sample was examined for total serum protein, serum protein fraction percentages and concentrations, alkaline phosphatase activity and serum concentrations of Ca^{++} and Mg^{++}.

Statistical analysis

All data were subjected to analysis of variance. Tables illustrating results of statistical analyses were constructed. Graphs and tables of mean values were constructed where appropriate.

PRESENTATION OF DATA

General observations

In the initial experiment, lasting 12 hours, there was no evidence of distress induced by the fluoride ion in any fish. Fish in some aquaria exhibited aggressive behavior toward their tank-mate initially; however, this ceased after an hour, or so, and normal, quiescent behavior was then observed. This initial reaction is presumed to mark adjustment of the trout to the smaller 20-gallon aquaria following transfer from the 300-gallon holding tanks. There was one death during the 12-hour experiment. This death was attributed to handling rather than to fluoride intoxication, for the trout had escaped and fallen to the floor several times during transfer from holding tank to aquarium. At 12 hours, all fish looked and acted as they had at the initiation of the experiment.

The first signs of distress appeared at approximately the eighteenth hour of the 24-hour experiment. It is presumed that some trout were naturally more susceptible to early effects of the fluoride ion and that more susceptible fish are the first to show distress. At 18 hours, an abundance of mucus was observed in most of those aquaria containing fish which later developed symptoms of acute fluorosis. In the final hours of the 24-hour experiment, those trout which had appeared to secrete an excess of mucus began to exhibit mild tetanus in fluoride ion concentrations of 2, 4, 7, 13,

and 25 ppm of F^-. Those trout exhibiting periodically recurring tetanus generally rested on their dorsal surfaces on the bottoms of the aquaria during quiescent periods between which were short intervals of rapid, nondirected darting about in the tanks. In the 24-hour experiment, one death occurred in an aquarium containing 13 ppm of F^-. This death was attributed to fluoride intoxication because the fish had exhibited symptoms indicative of fluorosis.

Fish that rested on the bottom of the aquaria between intermittent periods of tetanus were examined grossly and several interesting observations were noted. There was, generally, a grayish discoloration of the epidermis which commenced posteriorly on the peduncle and spread anteriorly to a general area immediately posterior to the dorsal fin. The discolored areas were considerably less slippery than the normally colored areas of the skin of afflicted fish. This condition was attributed to a considerably decreased rate of mucus secretion by mucous cells of the discolored areas of the epidermis. In addition, the muscle tissue underlying the discolored areas of the peduncle felt unusually firm. The entire peduncle was considerably less flexible in afflicted trout than it was in nonafflicted fish. Upon decaudation of these trout, it was observed that the amount of blood which could be obtained was always 50 percent, or less, than that obtained from trout not having the discolored, turgid peduncle. It was also noticed, upon decaudation of afflicted trout, that muscle tissue along the line of the cut tended to bulge outward. This condition was not observed in the nonafflicted trout. These effects were noted in more than half of the trout that exhibited symptoms of acute fluoride intoxication.

In the 36-hour experiment, some trout in the higher fluoride ion concentrations exhibited symptoms of fluoride intoxication at approximately the same time as similar symptoms were observed to develop in the 24-hour experiment. Throughout the remainder of all experiments, the symptom expression sequences were similar in both time and degree to those of earlier experiments. In the 36-hour experiment, at 26 hours symptoms of fluorosis became more pronounced in trout in aquaria containing 13 and 25 ppm F^-. After 29 hours, symptom expression was observed to extend down into fluoride ion levels of 7 ppm. At 34 hours there were 2 deaths in different aquaria containing 25 ppm F^-. Symptom expression had extended down into all concentrations of fluoride ion, but not in controls. Several trout in aquaria containing 2 ppm of fluoride ion were observed to exhibit the erratic behavior and tetanus that were evident in some trout in higher concentrations of fluoride ion. One death had occurred in an aquarium containing 13 ppm of fluoride ion at 24 hours, and one in the control group. The former death was attributed to fluoride intoxication because the fish had exhibited symptoms of fluorosis. The death of the control animal was known to have been brought about by excessive handling during transfer from the holding tank.

There was no evidence of the symptom expression discussed above in any control animals at any time during the course of all experiments. At the termination of the 36-hour experiment, one death had occurred in an aquarium holding 2 ppm of fluoride ion. Preliminary symptoms of fluorosis, including excessive mucus secretion, were observed in some fish in all fluoride ion concentrations, but not in control animals. In the final hours of the 36-hour run, behavior not observed in earlier experimentation was noticed in some trout.

exhibiting symptoms of acute fluorosis. Accompanying the tetanus and intermittent quiescent intervals, were periods during which there was an unusually rapid movement of the opercula and a tendency of afflicted fish to move backwards, along the bottom of the aquaria, on the tips of the pectoral and pelvic fins. This did not occur in control animals.

In the 48- and 60-hour experiments, symptom expression, when occurring, became more pronounced with the length of time that fish were exposed to any level of fluoride ion. Mucus secretion appeared to fall off in those fish having the discolored, firm peduncle. In some of the trout which did not exhibit outward signs of distress at any time, mucus secretion, as judged by accumulation of the secretion in the water and on the surface of the water, appeared to occur at considerably greater than normal rates.

In those experiments extending for 24 hours, or longer, development of the discolored, firm, and rigid peduncle effect was preceeded by a period of excessive mucus secretion at approximately 18 hours, In less susceptible trout, symptom expression was delayed for varied time intervals, depending upon the degree of susceptibility of the animal to fluoride ion. The discolored peduncle complex was observed in most of those trout which developed symptoms of acute fluorosis at a later time. The discolored peduncle complex, when occurring, persisted through all subsequent stages of intoxication.

Total serum proteins

The concentration of total protein in the serum of control and experimental rainbow trout was found to range between 1.92 gm./100 ml. and 4.77 gm./100 ml., with a mean for all trout through all

experimentation of 3.21 gm./100 ml.

Total serum protein concentrations (see Table 1) were found to vary, at the 95 percent level of confidence, with respect to the length of time trout were under experimentation. Effects of fluoride ion concentrations on serum protein levels were not significant statistically.

Figure 1 shows that there was a general decline in total serum protein concentrations of control animals through all hours of experimentation, with the exception of a rise at 48 hours. Serum protein concentrations of trout in all fluoride ion levels were lower than those of control animals between 12 and 24 hours, but were consistently higher than control values at 60 hours. It will be observed (see Figure 1) that at 60 hours the serum protein levels in control animals are considerably lower than serum protein levels of trout in all fluoride ion concentrations.

Interaction of time and fluoride ion concentration was significant at the 99 percent level of confidence (see Table 1). This indicates that particular combinations of time and fluoride ion concentration (see Table 2) have effects on total serum protein concentrations of fluoridated trout. These highly significant effects are not produced by time and fluoride ion concentrations independently, or, by various other combinations of time and concentration. Figure 1 illustrates the occurrence of some interaction between time and fluoride ion concentration at 24 hours in 4 ppm of F^- and at 48 hours in 7 ppm of F^-. Interaction produces the most significant variation of total serum protein levels of control trout from experimental animals between 48 and 60 hours. Serum protein

Table 1. Analysis of variance of total serum protein concentrations of the rainbow trout subjected to varied levels of fluoride ion for various periods of time

Source	Degrees of freedom	Sum of squares	Mean square	F Ratio
Total	59	35.1290		
Replications	1	0.8967	0.8967	2.683
Time	4	9.1552	2.2888	6.848*
Error (a)	4	1.3369	0.3342	
Concentration	5	0.3862	0.0772	1.643
Interaction	20	22.1782	1.1089	23.594**
Error (b)	25	1.1758		

*Significant at the 95 percent level of confidence.
**Significant at the 99 percent level of confidence.

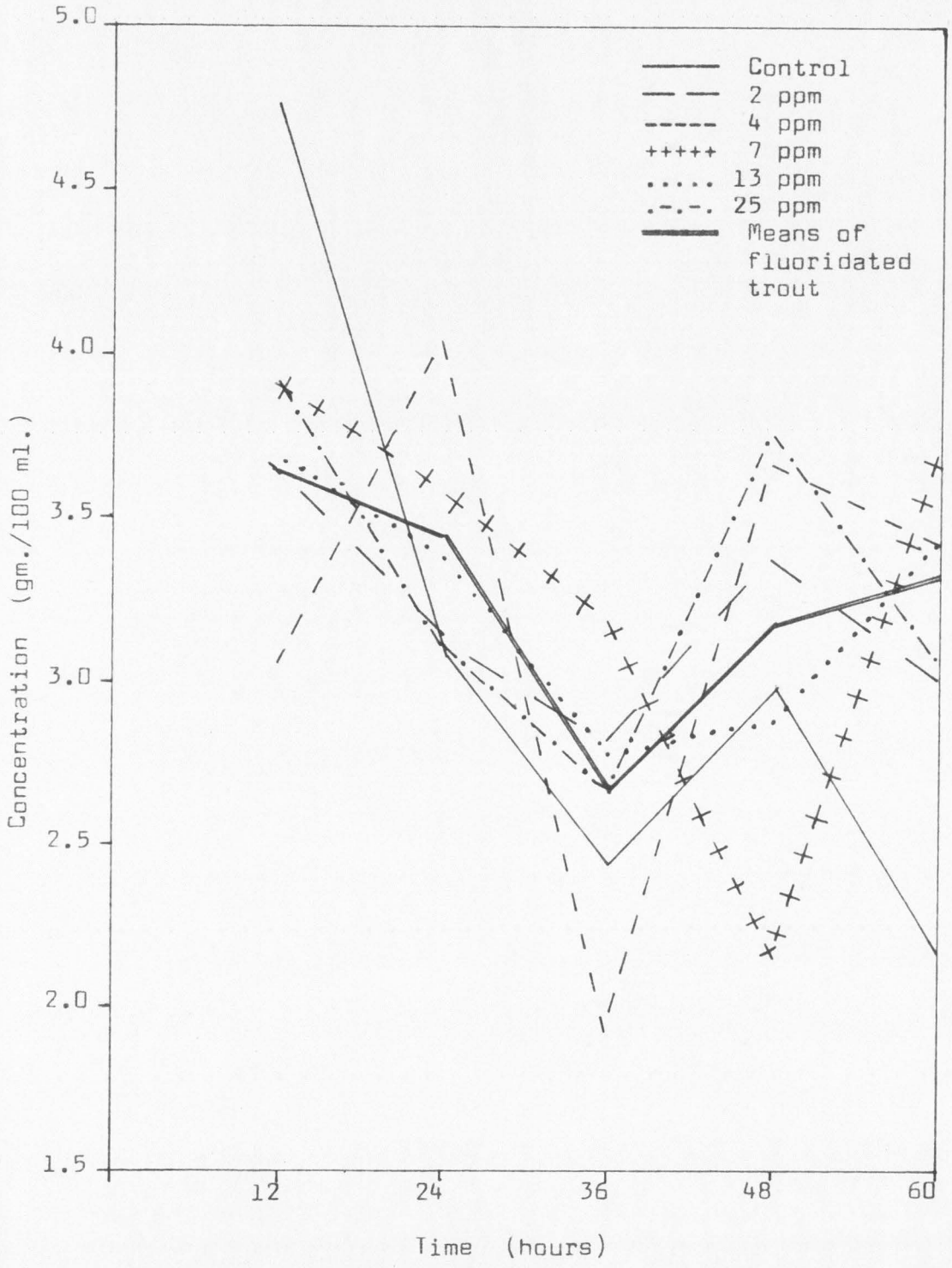

Figure 1. The relationship between total protein concentrations of the serum of control and fluoridated rainbow trout and length of the period of exposure to conditions of the experiment.

Table 2. Table of mean values of total serum protein concentrations of the rainbow trout subjected to varied levels of fluoride ion for various periods of time

Fluoride ion concentration (ppm)	Time (in hours)					Mean
	12	24	36	48	60	
0.2	4.77	3.10	2.43	2.98	2.17	3.09
2	3.66	3.13	2.80	3.38	3.00	3.20
4	3.05	4.04	1.92	3.66	3.41	3.21
7	3.90	3.58	3.18	2.14	3.69	3.30
13	3.68	3.38	2.79	2.87	3.41	3.22
25	3.90	3.12	2.66	3.75	3.05	3.30
Mean	3.82	3.39	2.63	3.11	3.12	3.21

concentrations of control animals are considerably lower at 60 hours than the levels in all fluoridated trout.

Serum protein fractions

Five protein fractions were identified in the serum of rainbow trout: gamma-globulin, beta-globulin, alpha$_2$-globulin, alpha$_1$-globulin, and albumin, the electrophoretic mobilities of each fraction increasing in the order given.

Serum fractions identified as gamma-globulin, beta-globulin, and alpha$_2$-globulin (see Table 3) were found to vary significantly, at the 95 percent level of confidence, with respect to the length of time that both control and experimental animals were under experimentation. No significant variation between levels of these serum fractions in control trout and in trout subjected to fluoride ion concentrations were found.

The concentrations of gamma-globulin (see Figure 2) in both control and experimental animals were observed to drop during all experiments. Between 12 and 24 hours, the level of gamma-globulin in control trout falls below that of fluoridated trout and remain lower than all subsequent values for gamma-globulin in fluoridated trout. Control and experimental levels of beta-globulin remains similar through all experiments, with the beta-globulin level of fluoridated trout maintaining a slightly higher value than that of control animals after 24 hours. The levels of alpha$_2$-globulin were more variable between control and experimental animals. Figure 2 shows that a degree of time and concentration interaction may have occurred at approximately 24 hours, for variance between control and fluoridated trout are greatest (see Figure 2) at that time. In all

Table 3. Analysis of variances of five serum protein fraction concentrations of rainbow trout subjected to varied levels of fluoride ion for various periods of time

Protein designation	Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio
Gamma-globulin	Total	59	0.565		
	Replications	1	0.012	0.012	4.00
	Time	4	0.202	0.050	16.66*
	Error (a)	4	0.011	0.003	
	Concentrations	5	0.010	0.002	0.25
	Interaction	20	0.118	0.006	0.75
	Error (b)	25	0.212	0.008	
Beta-globulin	Total	59	1.278		
	Replications	1	0.009	0.009	1.12
	Time	4	0.449	0.112	14.00*
	Error (a)	4	0.033	0.008	
	Concentrations	5	0.091	0.018	0.90
	Interaction	20	0.198	0.010	0.50
	Error (b)	25	0.498	0.020	
$Alpha_2$-globulin	Total	59	1.689		
	Replications	1	0.111	0.111	13.88*
	Time	4	0.331	0.083	10.38*
	Error (a)	4	0.030	0.008	
	Concentrations	5	0.018	0.003	0.08
	Interaction	20	0.550	0.016	0.45
	Error (b)	25	0.649	0.035	
$Alpha_1$-globulin	Total	59	4.845		
	Replications	1	0.010	0.010	0.23
	Time	4	0.803	0.201	4.67
	Error (a)	4	0.172	0.043	
	Concentrations	5	0.148	0.029	0.44
	Interaction	20	2.067	0.103	1.56
	Error (b)	25	1.645	0.066	
Albumin	Total	59	5.763		
	Replications	1	0.083	0.083	1.43
	Time	4	0.753	0.188	3.24
	Error (a)	4	0.234	0.058	
	Concentrations	5	0.362	0.072	1.28
	Interaction	20	2.929	0.146	2.61*
	Error (b)	25	1.402	0.056	

*Significant at the 95 percent level of confidence.

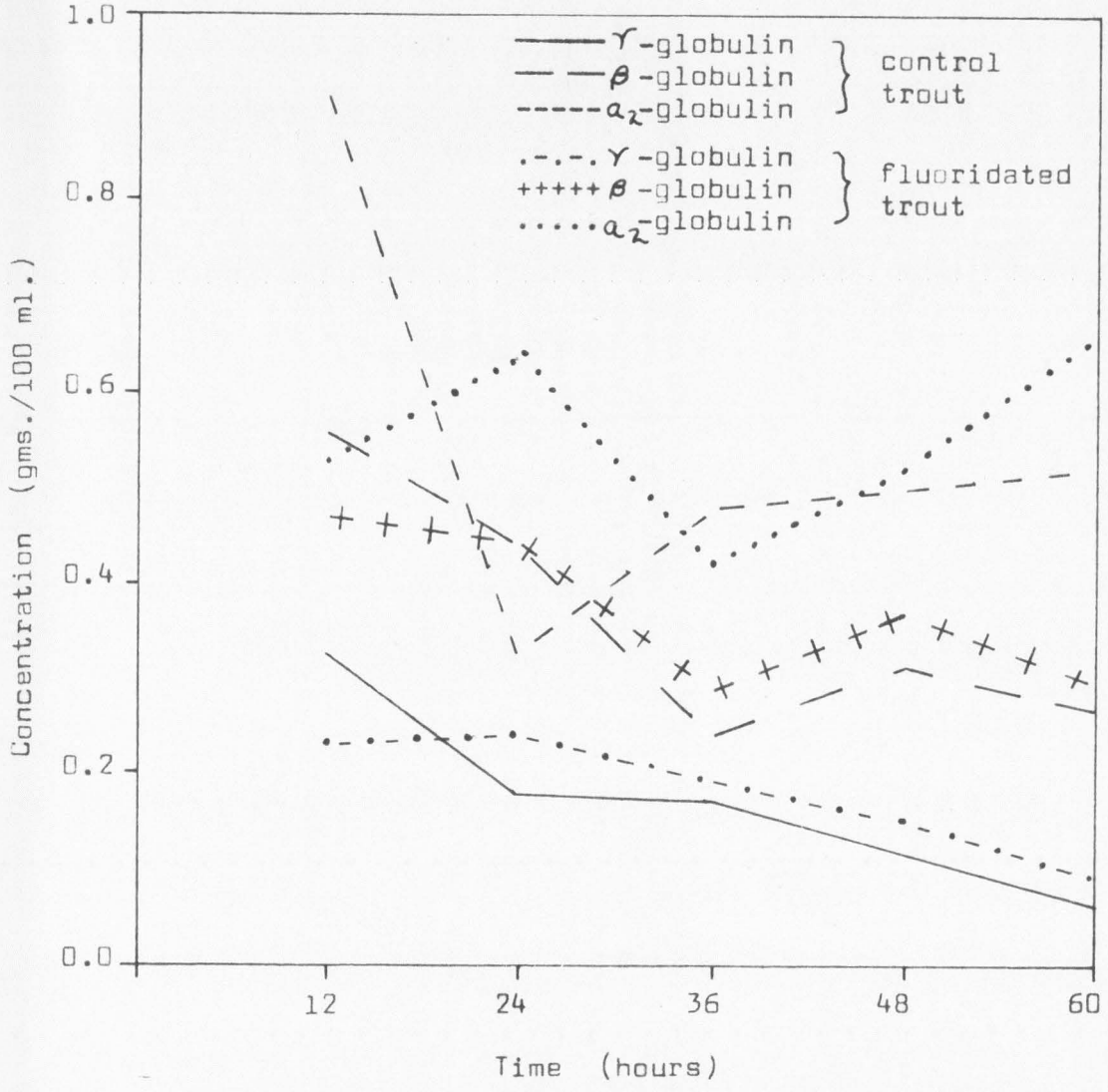

Figure 2. The relationship between concentrations of serum gamma-, beta- and alpha$_2$-globulin in rainbow trout and time the trout were exposed to the control medium and to various fluoride ion levels.

cases, as was found with total serum protein levels, concentrations of the gamma-, beta-, and alpha$_2$-globulins were lower at 12 hours than control levels, but were higher at 60 hours than the levels of these fractions in control animals.

Serum albumin levels (see Table 3) were found to vary, at the 95 percent level of confidence, with respect to interaction of time and fluoride ion concentration. Thus, particular combinations of time and fluoride ion concentration of the medium (see Table 4) produced variation in levels of serum albumin that were not produced by either time or fluoride ion concentration acting independently of each other, or, by various other combinations of time and fluoride ion concentration. Albumin levels have a downward trend through all hours of experimentation.

Serum alkaline phosphatase activity

Serum alkaline phosphatase activity in fluoridated rainbow trout was found to vary significantly, at the 99 percent level of confidence, with the concentration of fluoride ion in the experimental medium (see Table 5). Interaction of time and concentration, also at the 99 percent level of confidence, produced significant effects on alkaline phosphatase activity. No significant variation was found for alkaline phosphatase activity with respect to the time the trout were exposed to fluoride ion concentrations in the experimental medium.

Alkaline phosphatase in the serum of control fish had a mean value of 1.23 mM./L./hr. for all experiments, with no significant variation with respect to time. There was a steady decline (see Figure 3) in alkaline phosphatase activity in fluoride ion concentrations of 2 and 4 ppm. Activity increased steadily in fluoride

Table 4. Table of mean values of serum albumin concentration, expressed in gm./100 ml., of the rainbow trout subjected to varied levels of fluoride ion for various periods of time

	Time (in hours)					
	12	24	36	48	60	
Fluoride ion concentration (ppm)						Mean
0.2	1.50	1.21	0.73	0.97	0.50	0.98
2	1.18	0.83	0.99	1.40	1.25	1.13
4	1.06	1.46	0.64	0.84	0.98	1.00
7	1.36	1.10	1.18	0.73	1.31	1.14
13	1.07	1.20	1.07	1.02	1.18	1.11
25	1.56	0.85	1.06	1.35	1.16	1.20
Mean	1.29	1.11	0.94	1.05	1.06	1.09

Table 5. Analysis of variance of alkaline phosphatase activity of rainbow trout serum in varied concentrations of fluoride ion for various periods of time

Source	Degrees of freedom	Sum of squares	Mean square	F Ratio
Total	119	12.7070		
Replications	1	0.4521	0.452	1.222
Time	4	4.0783	1.019	2.754
Error (a)	4	1.4787	0.370	
Concentration	5	1.7246	0.345	6.273**
Interaction	20	3.5974	0.180	3.273**
Error (b)	25	1.3759	0.055	
Determinations	60	0.2330	0.004	

**Significant at the 99 percent level of confidence.

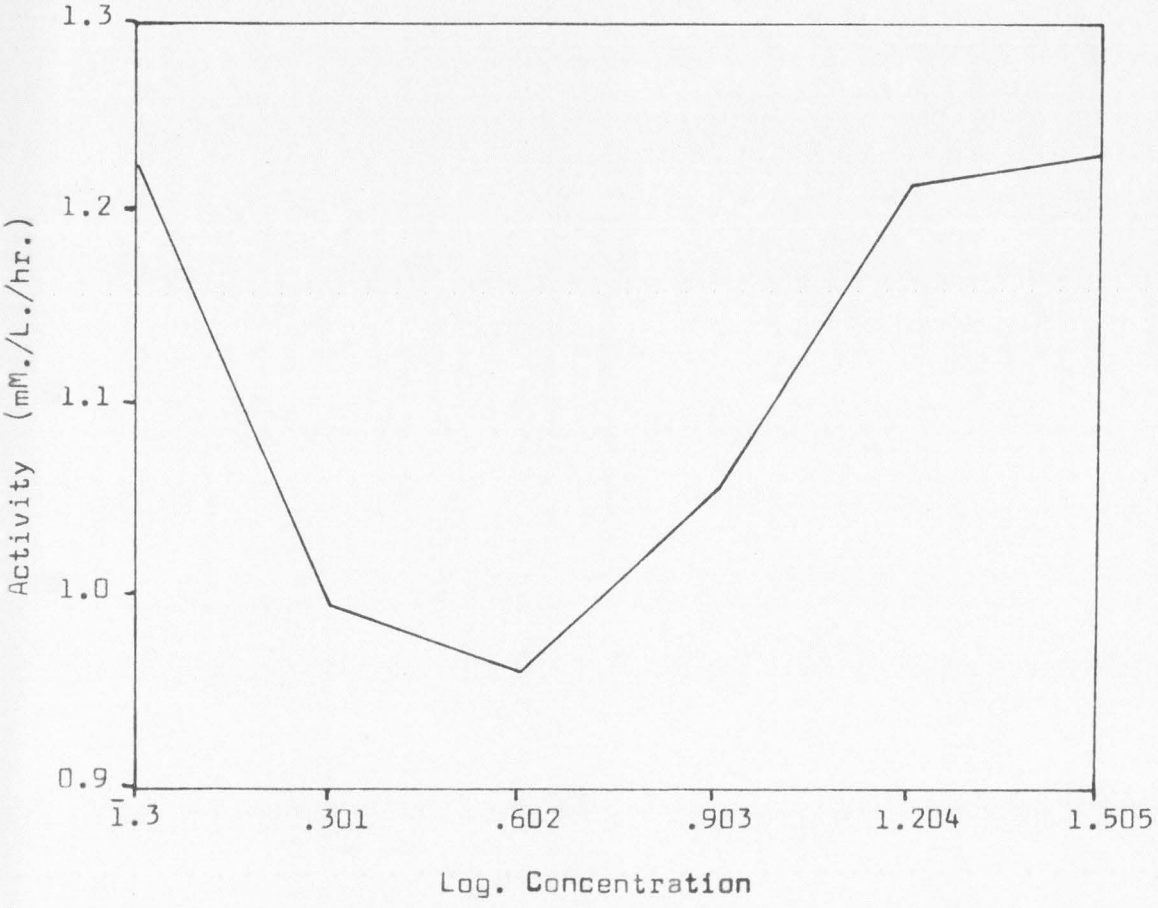

Figure 3. The relationship between fluoride ion concentration of the medium and alkaline phosphatase activity in trout serum.

ion concentrations above 4 ppm, with higher than control values occurring in fluoride ion concentrations of 25 ppm. Activity tended to level out between 13 and 25 ppm of F^-. Thus alkaline phosphatase activity was greater in trout exposed to 25 ppm of F^- than in control animals and animals subjected to 2, 4, and 7 ppm of F^-. Increased activity of the enzyme in higher fluoride ion concentrations was not as marked as the decrease in alkaline phosphatase activity which occurred in lower fluoride ion levels. Thus, it appears that lower fluoride ion levels exerted a depressing effect on alkaline phosphatase activity, while the converse occurred in higher fluoride concentrations.

Time and concentration interaction produced effects on serum alkaline phosphatase activity (see Table 6) which were not produced by the length of time the fish were exposed to any particular level of fluoride ion. Activity of this enzyme was lowest, as indicated by mean values, between 36 and 48 hours. The mean value for all concentrations of fluoride ion and all times is 1.12 mM./L./hr. and is only slightly lower than the mean activity value for all control animals.

Serum calcium

The concentration of serum calcium in fluoridated rainbow trout was found to vary significantly, at the 99 percent level of confidence, with the concentration of fluoride ion in the experimental medium (see Table 7 and Figure 4). Interaction of time and concentration, also at the 99 percent level of confidence, produced significant effects on serum calcium levels. There was no significant variation in serum calcium concentrations with respect

Table 6. Table of mean values of rainbow trout serum alkaline phosphatase activity, expressed in mM./L./hr., for fish in varied concentrations of fluoride ion for various periods of time.

	Time (in hours)					
	12	24	36	48	60	
Fluoride ion concentration (ppm)						Mean
0.2	1.35	1.39	1.33	1.13	0.99	1.225
2	0.95	1.42	0.88	0.68	1.05	0.999
4	0.81	1.25	0.93	0.84	0.96	0.960
7	0.80	1.64	0.79	0.82	1.23	1.057
13	0.95	1.40	1.12	0.85	1.74	1.212
25	1.32	1.30	0.93	1.18	1.65	1.270
Mean	1.03	1.40	0.99	0.91	1.27	1.120

Table 7. Analysis of variance of concentrations of calcium in the serum of trout subjected to varied levels of fluoride ion for various periods of time.

Source	Degrees of freedom	Sum of squares	Mean square	F Ratio
Total	59	40.727		
Replications	1	0.096	0.096	0.308
Time	4	1.539	0.385	1.230
Error (a)	4	1.252	0.313	
Concentration	5	7.365	1.473	4.332**
Interaction	20	21.963	1.098	3.229**
Error (b)	25	8.512	0.340	

**Significant at the 99 percent level of confidence.

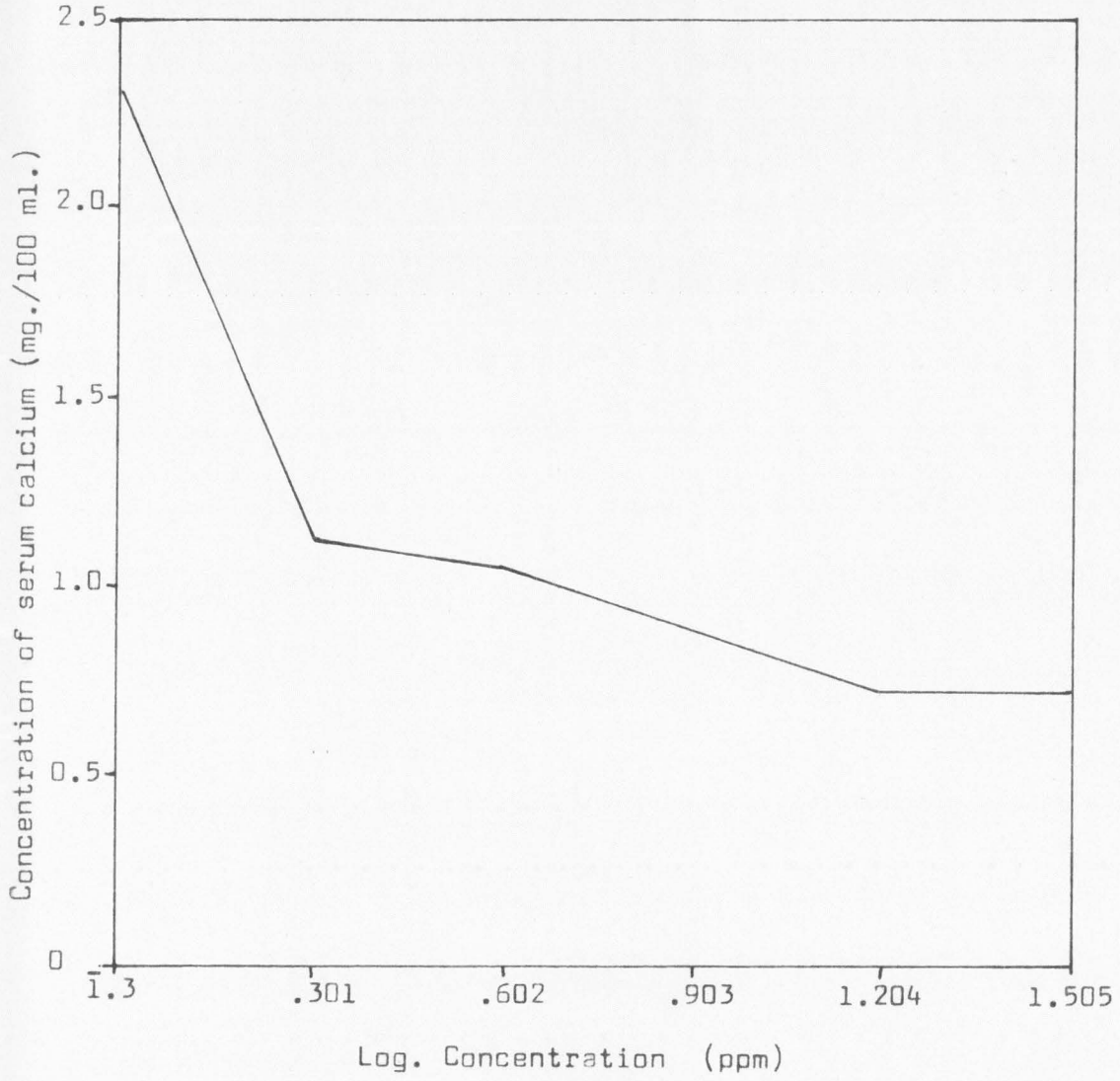

Figure 4. The relationship between the concentration of serum calcium of trout and fluoride ion concentration of the medium.

to the length of time the fish were exposed to concentrations of fluoride in the medium.

The mean value for serum calcium concentrations (see Table 8) in control trout was 2.22 mg./100 ml. The mean value for all experiments was 1.12 mg./100 ml., indicating that serum calcium concentrations decline considerably with fluoridation of the water. Table 8 shows that there is a steady lowering of serum calcium with respect to fluoride ion concentration of the medium (see Figure 4). There is also a decline in calcium levels with respect to time at a lower level of statistical significance.

Serum calcium declined from 2.22 mg./100 ml. in control trout to a mean of 0.72 mg./100 ml. in trout held in 25 ppm of fluoride ion (see Figure 4). A steady decline was noted through all concentrations of fluoride ion in the medium, the sharpest drop appearing in 2 ppm of fluoride ion. Between 2 ppm and 25 ppm the curve (Figure 4) shows that mean values of serum calcium decline gradually, reaching a low mean value of 0.72 mg./100 ml. in 13 ppm of fluoride ion and maintaining this value in 25 ppm F^-.

Time and concentration interaction produced effects on serum calcium concentrations (see Table 8) which were not produced by time and concentration of fluoride ion independently. While there was a marked decline in calcium concentration with respect to fluoride ion level of the medium, effects exerted by particular combinations of time and fluoride ion level were also highly significant. Table 8 shows that lower serum calcium concentrations are generally associated with higher fluoride ion levels through all experiments, although occasional low mean values for serum calcium

Table 8. Table of mean values of the concentration of calcium, expressed in mg./100 ml., in the serum of trout subjected to varied concentrations of fluoride ion for various periods of time.

	Time (in hours)					
	12	24	36	48	60	
Fluoride ion concentration (ppm)						Mean
0.2	1.9	3.5	3.3	1.5	0.9	2.22
2	1.7	0.8	0.8	1.3	1.1	1.14
4	1.4	1.2	0.6	0.6	1.4	1.04
7	1.1	0.6	1.0	0.8	1.0	0.90
13	1.2	0.5	0.7	0.6	0.6	0.72
25	1.1	0.8	0.6	0.7	0.4	0.72
Mean	1.40	1.23	1.17	0.92	0.90	1.12

appear in lower fluoride ion concentrations.

Serum magnesium

The concentration of serum magnesium in fluoridated trout was found to vary significantly (see Table 9), at the 95 percent level of confidence, with respect to concentration of fluoride ion in the experimental medium. Interaction of time and fluoride ion concentration produced significant variation, also at the 95 percent level of confidence, in serum magnesium concentrations. As with serum calcium, there was no highly significant effect of the exposure period of trout to fluoride ion concentrations.

Figure 5 illustrates the rise in serum magnesium concentrations with respect to fluoride ion levels in the experimental medium. The mean value for magnesium concentration in control trout is 5.64 mg./100 ml. (see Table 10). There is a drop in serum magnesium concentration in 2 ppm F^- which corresponds to a drop in alkaline phosphatase activity in 2 ppm F^- (see Figure 3). Serum magnesium (Figure 5) increases in concentration in all fluoride ion levels above 2 ppm and in 25 ppm F^- has a mean value of 7.32 mg./100 ml. (see Table 10). This value is higher than serum magnesium concentrations in control animals and also correlates with increased alkaline phosphatase activity (Figure 3). There is also a gradual increase in serum magnesium concentrations with respect to the length of the period of exposure of trout to all fluoride ion levels in the experimental medium. This effect was statistically less significant than effects produced by fluoride ion levels or interaction.

Time and concentration interaction produced effects on serum

Table 9. Analysis of variance of concentrations of magnesium in the serum of rainbow trout subjected to varied levels of fluoride ion for various periods of time.

Source	Degrees of freedom	Sum of squares	Mean square	F Ratio
Total	59	423.52		
Replications	1	2.50	2.500	0.377
Time	4	89.12	22.280	3.356
Error (a)	4	26.55	6.638	
Concentration	5	47.95	9.590	2.846**
Interaction	20	173.18	8.659	2.570**
Error (b)	25	84.22	3.369	

**Significant at the 95 percent level of confidence.

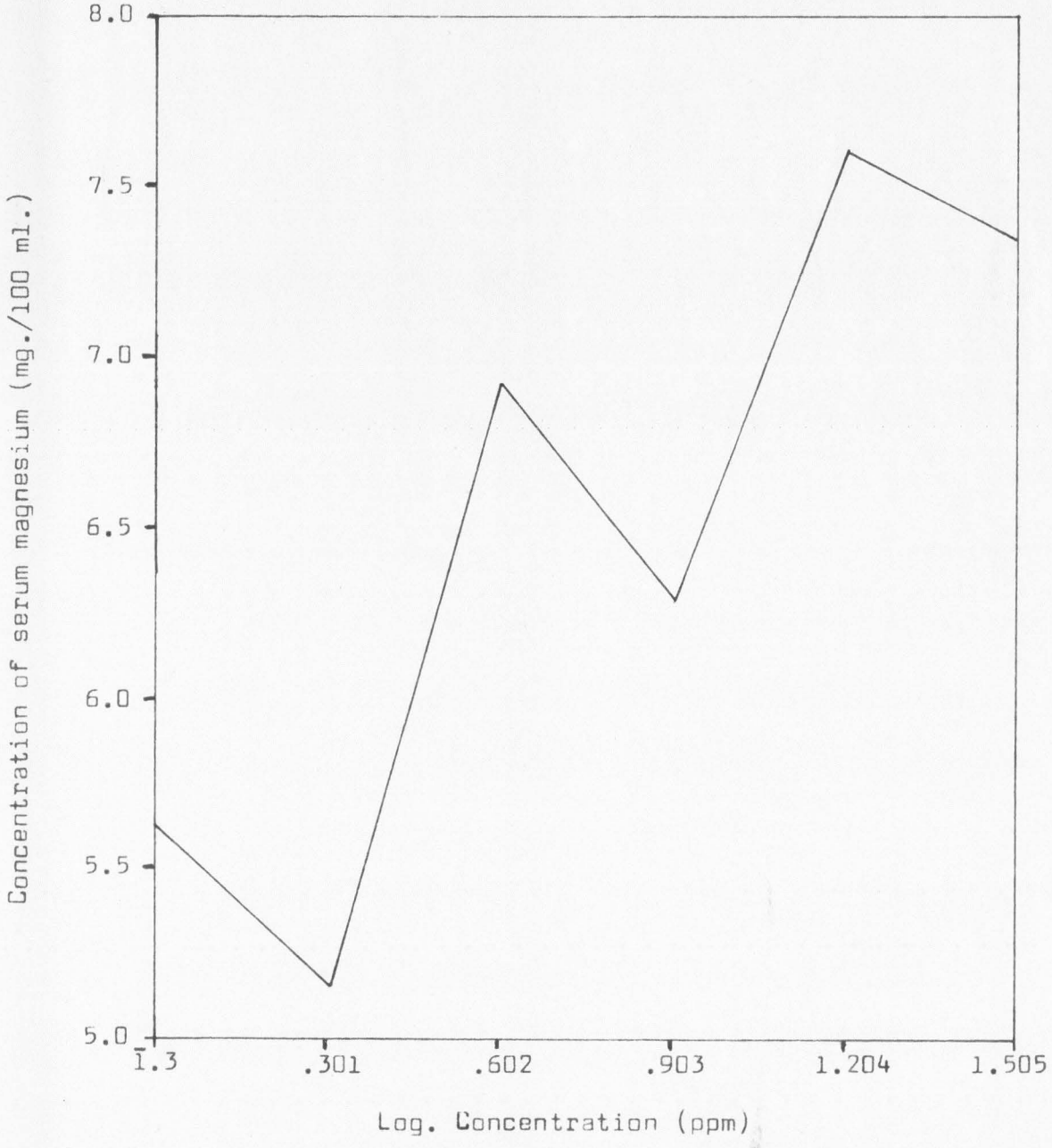

Figure 5. The relationship between the concentration of serum magnesium of trout and fluoride ion concentration of the medium.

Table 10. Table of mean values of the concentration of magnesium, expressed in mg./100 ml., in the serum of rainbow trout subjected to various concentrations of fluoride ion for different periods of time.

	Time (in hours)					
	12	24	36	48	60	
Fluoride ion concentration (ppm)						Mean
0.2	3.3	6.7	4.7	6.8	6.7	5.64
2	3.8	1.8	8.3	5.7	6.3	5.18
4	2.4	5.2	9.8	8.9	8.2	6.90
7	3.3	7.4	5.5	6.8	8.5	6.30
13	8.9	8.7	8.8	6.3	5.3	7.60
25	3.7	8.0	5.1	9.5	10.3	7.32
Mean	4.23	6.30	7.03	7.33	7.55	6.49

magnesium levels that were independent of those effects caused by time and fluoride ion.

DISCUSSION

Total serum protein levels

There were no highly significant differences in levels of total serum protein in rainbow trout with respect to fluoride ion concentration of the medium independently. Both control and fluoridated trout (see Figure 1) were shown to have serum protein levels which vary significantly with the length of the time that the fish are exposed to conditions of the experiments. Since the decline in serum protein concentration cannot be attributed to fluoride ion, this effect must be caused by the action or actions of other factors. The possibility that increased capillary permeability caused by fluoride ion (Sollmann, 1948) may result in movement of plasma proteins into intercellular spaces is appealing. However, this explanation does not consider parallel declines in total serum protein levels in control animals. It may be postulated that sudden change in the environment, coupled with confinement of the animal to small areas, may contribute in some manner to the serum protein complex observed in my study. The nature of the mechanisms that operate herein are obscure.

It was found that (see Table 1 and Figure 1) interaction of time and fluoride ion concentration produced highly significant effects on total serum protein levels. Interaction is greatest between 48 and 60 hours. Within this time interval, the means of the serum proteins of fluoridated trout rose to 3.3 gm./100 ml. while the levels in control animals declined steadily to a mean of 2.16 gm./100 ml.

While it has been admitted that reasons for the lack of significant effects of fluoride ion on serum protein levels with regard to fluoride concentrations independently are obscure, it is possible to postulate certain mechanisms which may have operated in interaction.

The observed reduction in blood volume is suggestive of movement of blood fluids into intercellular and intracellular areas, resulting in a hemoconcentration. If this actually occurs, there would be a tendency toward concentration of plasma proteins, an effect which appears to occur after 48 hours in fluoridated trout, but not in control animals. Sollmann (1948) reports that fluoride ion increases permeability of capillary walls to plasma fluids. Why a rise in plasma proteins does not occur in response to fluoride ion earlier is difficult to understand. It may be that full effects of absorbed fluoride do not occur until after trout have been exposed to the fluoridated medium for approximately 48 hours, the time when interaction becomes particularly significant.

Plasma protein fraction levels

Decline in the fractions of gamma-, beta-, and $alpha_2$-globulins with respect to the length of time that both control and fluoridated trout were exposed to conditions of the experiments was significant. Effects of fluoride ion on levels of these fractions was not significant. Again, reasons for this condition are difficult to comprehend. Since albumin has a smaller molecule than the globulins, it would seem that a decline in albumin would accompany the decline in globulins. This was not the case. It may be that the mechanisms which operate within this complex are associated, in some manner,

with a radical change in environment of the trout and concordant confinement of the animal to very restricted areas.

Significant variation in albumin levels was found with respect to interaction of time and fluoride ion concentration. It will be recalled that excess mucus secretion was observed to occur in more than half of those trout which developed symptoms of acute fluoride intoxication. Mucus is composed principally of albumin (Brown, 1957), and is secreted by mucous cells lying in the epidermis of the fish. De Roos (1958) found that in goldfish there is an increase in the number of mucous cells on the gill lamellae in response to elevated levels of fluoride ion in the environment. Neuhold (1959) found an increase in density of mucous cells in the head region of rainbow trout exposed to fluorides. The skin of the trout has been shown to excrete chlorides (Brown, 1957). Therefore, it is suggested that there may be some relationship between mucus secretion and lowered albumin levels, with the possibility that fluoride ion excretion may be associated in some manner. It is well known that fluoride ion increases permeability of capillary walls. Therefore, since albumin is the smallest of plasma protein molecules, it may be that albumin passes from blood capillaries into mucous cells. That chlorides may be eliminated through the skin of the trout has been mentioned. Albumin could conceivably form a complex with electronegative fluoride ion and thus be eliminated from the fish in mucus. This is a postulated mechanism and its occurrence remains to be established.

Also, it is reported that fluoride ion increases the permeability of the kidney tubules to albumin (Sollmann, 1948), with resultant

albuminuria. Loss of albumin may result in a decreased blood volume, since this fraction is more concerned with the maintenance of an osmotic gradient between the blood and intercellular areas (Best and Taylor, 1955) than are other fractions. Decreased blood volume was observed in over half of the trout exhibiting the discolored peduncle, and did not occur in controls. Why lowered albumin occurs only in particular combinations of time and fluoride ion concentration, and not in all concentrations at all times is certainly not clear. Also, albumin was observed to decline considerably in control animals in longer experiments, a fact which confounds explanation.

Blood alkaline phosphatase activity and levels of calcium and magnesium

Phillips (1932) found that plasma alkaline phosphatase activity in fluoridated bovines increases in proportion to the level of fluoride. Phillips (1934) postulates that increased alkaline phosphatase activity is the mechanism whereby fluorides are removed from systemic circulation and deposited in osseous tissues of the fluoridated animal. Neuhold (1959) found that fluoride content of the bones of rainbow trout subjected to different fluoride ion concentrations is proportional to fluoride ion concentration of the medium. Since alkaline phosphatase activity in my experiments was found to rise in fluoride ion levels higher than 4 ppm, it is postulated that the increased activity of the enzyme is the mechanism whereby fluoride may be deposited in osseous tissue of the fluoridated animal. It has been well established that alkaline phosphatase is closely associated with

calcium and phosphate metabolism and bone production (Sumner and Myrback, 1951; Hawk et al., 1951; Best and Taylor, 1955).

Figure 3 shows that serum alkaline phosphatase activity in the fluoridated trout decreases in fluoride ion concentrations of 2 ppm and 4 ppm and increases in all higher fluoride ion levels to a higher than normal value in 25 ppm F^-. Figure 5 shows that serum magnesium concentrations also decline in 2 ppm F^- and have higher than normal values in all fluoride ion levels above 2 ppm F^-. Since Mg^{++} has been found to be necessary for activation of alkaline phosphatase, it appears that increased magnesium concentrations in the higher fluoride ion levels may be associated with the increased alkaline phosphatase activity as shown by the data.

The initial drop of alkaline phosphatase activity in lower fluoride ion concentrations, accompanied by low serum magnesium levels, may indicate that low fluoride levels are not sufficiently toxic to the fish to cause increased alkaline phosphatase activity. It is also possible that fluoride ion can be effectively eliminated from trout as a mucus-fluoride complex in lower fluoride levels. As the fluoride level of the medium becomes more concentrated, it may be postulated that the efficiency of excretion of circulating fluoride is diminished and that increased alkaline phosphatase activity of animals in the higher fluoride ion concentrations marks an additional mechanism whereby fluorides are rendered less harmful to the organism. The increased fluoride content of bone tissue of fluoridated trout which is proportional to the fluoride ion concentration of the medium (Neuhold, 1959) appears to lend support to the possibility of increased alkaline phosphatase activity

having close association with fluoride deposition in bone tissue.

The source of increased plasma magnesium in the fluoride-treated trout remains obscure. L. E. Olson[1] reports that serum magnesium levels in fluoridated cattle rise proportionally with the fluoride concentration ingested. Magnesium was not absorbed by trout from the experimental medium because all calcium and magnesium ions were removed from the water by the ion exchange resin employed for that purpose. Most of the magnesium present in higher vertebrates is found in the bones (Bell et al., 1957). It is possible, therefore, that magnesium stores in osseous tissues are mobilized in the fluoridated animal. This would account for increased serum magnesium concentrations indicated by the data.

The decline in plasma calcium concentrations in the fluoridated trout could be brought about in a number of ways. As increased alkaline phosphatase activity in fluoridated trout may mark deposition of fluorides in osseous tissue. Neuman et al. (1950) report that fluoride ion may substitute for hydroxyl and bicarbonate radicals on the surface of osseous tissue, forming a very stable union with calcium. This would reduce plasma calcium concentrations. Turner (1955) indicates that hypocalcemia and hypertrophy of the parathyroids occur in hypoparathyroidism. Neuhold (1959) found hypertrophy of the ultimobranchial glands of fluoridated rainbow trout and postulated that a calcium deficiency is associated with this condition. Tetanus was observed in fluoride intoxicated rainbow trout by Neuhold (op. cit.) and was also characteristic of acutely

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[1]Personal communication.

fluoridated trout in my experiments. Tetanus may indicate calcium deficiency (Turner, 1955).

It appears that magnesium and calcium metabolism are closely associated with alkaline phosphatase activity of the fluoridated trout. The rise in alkaline phosphatase activity, accompanied by increased concentration of Mg^{++}, a phosphatase activator, a decreased plasma calcium level accompanied by tetanus and increased fluoride deposition in bone tissue and hypertrophy of the ultimobranchial glands are certainly indicative of a close correlation between fluoride ion concentration of the medium and the osseous metabolism of the fluoridated trout.

SUMMARY

A total of 240 rainbow trout, each approximately 10 inches in length, were subjected to fluoride ion concentrations of 0.2, 2, 4, 7, 13, and 25 ppm. In all, 5 experiments were performed, each utilizing 48 trout. The initial experiment lasted 12 hours, each additional experiment lasting 12 hours longer than the one preceeding it. All experiments were conducted in walk-in type cooling units at a maintained temperature of 55° F, maximum variation being ± 1° F. Samples of blood were obtained from each trout at the termination of each experiment. Sixty serum samples were selected at random from control and fluoridated animals and were analyzed for alkaline phosphatase activity, concentrations of serum Mg^{++} and Ca^{++}, total serum protein and changes in the percentage and amount of serum protein fractions.

1. Total serum proteins were found to fall in all experiments in both control and fluoridated trout with respect to time the fish were under conditions of the experiments. No significant differences between the total serum protein levels of control and fluoridated trout could be attributed to the independent action of fluoride ion. It is suggested that a radical change in environment with accompanying confinement of the trout to very restricted areas affect plasma protein levels. Interaction was highly significant.

2. Five protein fractions were identified in the serum of the rainbow trout. The gamma- and beta-globulins of both control and

fluoridated trout varied significantly with time, but were independent of fluoride ion concentration. Albumin was variable through the combined effects of interaction. It is postulated that decreased plasma albumin may be associated with mucus secretion and fluoride ion elimination from the fluoridated trout.

3. Plasma magnesium was found to rise significantly in fluoridated trout, following a decline in lower fluoride ion concentrations. Magnesium variation was significant with respect to fluoride ion concentration of the experimental medium, and with respect to interaction.

4. Plasma calcium variations were highly significant with respect to fluoride ion concentration of the medium and with respect to interaction. Plasma calcium levels were observed to decline markedly in fluoridated trout. Tetanus was observed frequently in the fluoridated trout and was associated with calcium deficiency.

5. Alkaline phosphatase activity was found to fall in lower fluoride ion levels, and to rise to higher than normal values in 25 ppm of fluoride ion. It is postulated that there is a relationship between alkaline phosphatase activity, plasma calcium and magnesium, hypertrophy of the ultimobranchial gland, and osseous metabolism in fluoridated rainbow trout.

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